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# Closely related intertidal and deep-sea *Halomonhystera* species have distinct fatty acid compositions

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## Abstract

The deep-sea free-living nematode *Halomonhystera hermes*, dominant in the sulphidic sediments of the Håkon Mosby mud volcano (1280 m, Barent sea slope), is part of the mainly estuarine *Halomonhystera disjuncta* species complex consisting of five cryptic species (GD1–GD5). Cryptic species have a very similar morphology raising questions on their specific environmental differences. This study analyzed total fatty acid (FA) compositions of *H. hermes* and GD1, one of *H. hermes*'s closest relatives. Additionally, we experimentally investigated the effect of a temperature reduction, salinity increase and sulphide concentrations on GD1's FA composition. Because nematodes are expected to have low amounts of storage FA, total FA compositions most likely reflect FA contents of cellular membranes. The deep-sea nematode *H. hermes* had significantly lower saturation levels and increased highly unsaturated fatty acid (HUFAs) proportions due to the presence of docosahexanoic acid (DHA—22:6 $\omega$ 3) and higher eicosapentaenoic acid (EPA—20:5 $\omega$ 3) proportions. HUFAs were absent in *H. hermes*'s food source indicating the ability and need for this nematode to synthesize HUFAs in a deep-sea environment. Our experimental data revealed that only a decrease in temperature resulted in lower saturated fatty acids proportions, indicating that the FA content of *H. hermes* is most likely a response to temperature but not to sulphide concentrations or salinity differences. In experimental nematodes, EPA proportions were low and DHA was absent indicating that other factors than temperature, salinity and sulphides mediate the presence of these HUFAs in *H. hermes*.

**Keywords:** Nematodes, Deep sea, *Halomonhystera*, Fatty acids, DHA, Hakon mosby mud volcano

## Background

Organisms require adaptive responses when presented to changes within their environment to survive and reproduce successfully. Environmental adaptation can result in morphological, physiological, behavioural or developmental differences. It is known that environmental changes can alter cell membrane fluidity, which could impede membrane function and stability, and performance of membrane-associated enzymes [61]. Cellular membranes function as a physiological barrier with a limited permeability and mainly consist of phospholipids, of which fatty acids are the most variable part

[40]. It is, therefore, essential that the liquid-crystalline structure of the membrane is maintained by regulation of the FA composition to acclimatize cells to changing environmental conditions, as is evidenced from temperature effects in poikilotherms [28]. Alterations in the composition of membrane phospholipids in response to environmental changes are of critical importance for the function and integrity of cell membranes, and is a general phenomenon in animals, known as “homeoviscous adaptation” (HVA) or “homeophasic adaptation” [30, 39, 61].

Nematodes, one of the most numerous of all Metazoa in terms of species richness [41], exist in all habitats that can support life and demonstrate remarkable abilities to withstand stress and adverse conditions [44, 62]. It has been suggested that marine nematodes do not accumulate lipids for energy storage [27] indicating that nematode fatty acid composition reflects membrane fatty acid

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composition, however, information remains scant. In this study we will focus on the marine free-living nematode *Halomonhystera disjuncta* (formerly known as *Geomonhystera disjuncta*), which is known for its high resistance to environmental stress, especially to heavy metals and temperature changes [25, 75–77]. *H. disjuncta* and sister species has been reported both in shallow-water [15, 47, 74] and in deep-sea environments [53, 71]. Mitochondrial and nuclear DNA sequences as well as morphometric analysis revealed that *H. disjuncta* from the Western Scheldt and the North Sea actually consist of five cryptic species (GD1–GD5) [15] with nearly imperceptible morphometric differences [22]. The same marine nematode has been morphologically identified as the dominant nematode species at the sulphide rich bacterial mats of two methane cold seeps: the Nyegga pockmarks (Nordic Norwegian margin, 730 m water depth) [53] and Håkon Mosby mud volcano (HMMV, Barents Sea slope, 1280 m water depth) [71]. The HMMV relative is closely related to the intertidal *Halomonhystera* cryptic species complex but can be distinguished from its shallow-water relatives both morphometrically and with the use of genetic markers [69], and was, therefore, described as a new species named *H. hermes*i [64]. Phylogenetic relationships showed that *H. hermes*i is most closely related to GD1 and GD4. Interestingly, GD1 appeared to be more resistant to simulated cold seep conditions compared to GD2–GD3 [68]. However, lack of any information on the biochemical resources (e.g. FA composition) hampers our understanding of the unique biology of closely related species in different environment.

The deep sea is a relatively harsh environment with constantly low ambient temperatures (~2 °C), high water pressure and absence of light. Furthermore, cold seeps are characterized by methane seepage through sediment and rely on chemosynthetically derived energy. They often have high concentrations of sulphides, as a result of microbial sulphate reduction coupled to anaerobic oxidation of methane [43]. Most studies on adaptive changes of the FA composition to deep-sea conditions have been performed on barotolerant bacteria under different experimental conditions and have revealed increased proportions of mono- and poly-unsaturated FAs in response to increased pressure and/or reduced temperature [13, 67, 80]. Consistent with bacterial responses, a comparison of mitochondrial membranes isolated from shallow and deep-sea fish species has identified similar changes in unsaturation levels [10, 73]. Moreover, highly unsaturated FAs (HUFAs) are believed to be essential in piezophilic adaptation of cellular membranes [81] and docosahexaenoic acid (DHA—C22:6 $\omega$ 3) was hypothesized to be the key to HVAs to depth and temperature changes in vertically migrating planktonic copepods [52].

As such, we expect higher proportions of unsaturated fatty acids and HUFAs in *H. hermes*i compared to GD1.

The second part of this study consists of a comparison of the FA composition of GD1 nematodes under simulated estuarine and bathyal seep conditions. We investigated the effect of three abiotic factors salinity, temperature and sulphide on total fatty acid composition. Each of the three abiotic factors varied from control conditions (salinity of 25, 16 °C and no sulphide) to environmental conditions representative of the HMMV (salinity of 34–35, –0.9 °C and hydrogen sulphide concentrations up to 1 mM) [58, 72]. As previously mentioned, temperature decrease has a clear effect on the FA composition in cellular membranes resulting in an increase in the unsaturated fatty acid proportions. Such a response is uniform and we, therefore, anticipate similar outcomes. The effect of increased salinity, however, is an increase of saturated FA levels and decreased proportions of unsaturated FAs as described for the bacteria *Shewanella* [49] and *Halomonas* species [37], and several plant species from the *Ephedra* genus [3], but information remains scant. Moreover, salinity effects were also found to be opposite between different plant varieties of the same species [34]. Lastly, no information on the effects of sulphides on FA composition currently exists. We can, however, consider sulphides to be a toxic compound because *H. disjuncta* is unable to survive concentrations higher than 1.5 mM (personal observations). In the presence of a toxic compound in sublethal concentrations, cells start to decrease membrane fluidity by increasing the degree of FA saturation [17, 48, 60]. The experimental data, discussed within this paper, allows investigating the individual effects of environmental factors on FA composition. In addition, it can aid in the correct interpretation of observed differences in FA composition between *H. hermes*i and GD1 sampled from the HMMV and Western Scheldt estuary, respectively.

## Methods

### Intertidal GD1 nematode cultures

Decaying *Fucus vesiculosus* was collected from the Paulina tidal flat, located along the southern shore of the polyhaline zone of the Western Scheldt estuary (southwestern part of The Netherlands). Macroalgal pieces were transferred to Petri dishes containing 0.8 % nutrient:bacto agar (ratio of 1/7) prepared in artificial seawater [46] with a salinity of 25 and placed at a constant temperature of 16 °C. Two weeks after inoculation, one single gravid female was transferred to a new Petri dish (5.5 cm inner diameter), containing the same agar medium, to establish monospecific GD1 cultures. Frozen-and-thawed *Escherichia coli* K12 was added as a food source and cultures were placed at a constant

temperature of 16 °C. Monospecificity and species identity were verified, shortly after inoculation, by PCR and sequencing the ITS gene. Primers and thermal-cycling conditions can be found in Derycke et al. [15].

### Sample collection

Intertidal GD1 nematodes were harvested from the monospecific cultures and transferred to rehydrated (salinity of 25), defaunated *F. vesiculosus* (48 h at 60 °C). They were allowed to grow for ca. 4 weeks in three litterbags (mesh size 200 µm) at 8 °C and a salinity of 25. Litterbags were then placed at the Paulina tidal flat (Western Scheldt estuary, 51°20'56.79"N, 3°43'29.56"E) for 72 h. Following this period, macroalgae were washed over two stacked sieves (top sieve: 1 mm, bottom sieve: 32 µm) and fauna was retained on the bottom sieve. The sieve was rinsed with sieved seawater (32 µm) and replicate samples were frozen in liquid nitrogen and stored at −80 °C awaiting further analysis. Throughout the rest of the study, GD1 samples extracted from the Paulina tidal flat, will be referred to as GD1<sub>p</sub>.

Deep-sea sediment samples were collected at the HMMV during the Maria S. Merian cruise 2010 using a TV-guided multi-corer. The microbial mats, the preferred habitat of *H. hermesii*, were selected and sampled from three locations (Station MSM16-2\_802-1, 72°0.17'N, 14°43.88'E; Station MSM16-2\_829-1, 72°0.16'N, 14°43.94'E; Station MSM16-2\_831-1, 72°0.14'N 14°43.94'E). Cores from these locations were immediately sliced on board from 0 to 10 cm with a one cm interval and samples for fatty acid composition were stored at −80 °C.

### Nematode extraction

The top cm of three deep-sea samples and part of the three intertidal samples, were thawed and nematodes were extracted by density gradient centrifugation, using Ludox (a colloidal silica polymer; specific gravity 1.18) as a flotation medium [31]. For each replicate, ca. 333 nematodes of each sex (male and female) and 333 juveniles were manually picked out using a binocular microscope to easily select *Halomonhystera* species based on their morphology. Per replicate, juveniles and adults were pooled to encompass all life stages and sexes present in the population. Nematodes were then rinsed twice in sterile Mili-Q for at least 1 h to remove most adhering bacteria and transferred to glass 5 ml GC vials in sterile Mili-Q and stored at −80 °C prior to fatty acid extraction.

### GD1 experimental set up

The effect of three abiotic factors (salinity, temperature and sulphide presence/absence) on fatty acid composition was tested by a replicated fully crossed design with

three factors: a salinity of 25 and 34, temperatures of 16 and 4 °C and presence or absence of sulphide (0 and 1 mM). Because simulated cold deep-sea temperatures resulted in occasional crystallization of the agar medium, a temperature of 4 °C was used. Experimental control conditions were 16 °C and a salinity of 25 because stock cultures are maintained at these conditions. The experiment was conducted on experimental microcosms which consisted of Petri dishes (5.5 cm i.d.) containing 1.5 ml 0.8 % nutrient:bacto agar (1/7). The sulphidic medium consisted of the same agar as described above to which we added sodium thiosulphate (Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) and sodium sulphide (Na<sub>2</sub>S) in a final concentration of 1 mM for both reagents; 350 µl *E. coli* K12 (ca.  $8 \times 10^8$  cells/ml) was added as a food source, sufficient for the duration of the experiment. In addition, 350 µl of the *E. coli* food source was also stored at −80 °C for further fatty acid analysis.

The experiment was started by manually picking out 60 females and 40 males (F<sub>0</sub>) from the monospecific GD1 stock cultures which were deposited in the microcosms. Petri dishes were closed with parafilm and maintained in temperature-controlled incubators without light for the whole length of the experiment. Two days after the minimum generation times (9–11, 10–15 and 28–39 days at 16, 10 and 4 °C, respectively) [68], 333 F<sub>1</sub> nematodes (111 juveniles, males and females) from each replicate were manually picked out and washed as described above. Because we required 1000 nematodes for further analysis and microcosms did not yield many nematodes, the replicate samples were pooled, frozen in 5 ml GC vials and stored at −80 °C.

### Analytic techniques and data treatment

Deep-sea, intertidal and experimental GD1 samples, and the *E. coli* food source were lyophilized for 24 h before extraction. Extraction and esterification of fatty acids was performed according to a modified one-step method of Abdulkadir and Tsuchiya [1]. Briefly, samples were mixed with 700 µl of a 2.5 % H<sub>2</sub>SO<sub>4</sub>–methanol solution in a capped tube and heated at 80 °C for 90 min. After cooling to room temperature, 350 µl hexane and 350 µl of a 0.95 % NaCl solution were added. After stirring, samples were centrifuged for 10 min at 1000 rpm (650 g). Of the two phases which formed, the upper hexane layer, containing the fatty acid methyl esters (FAMES), was transferred to a capped vial to be injected into the gas chromatographer (GC) for FAME analysis. Five microlitre of the FAMES solution was injected on a HP88 column (Agilent J&W) at a temperature of 250 °C and separated using gas chromatography (Hewlett Packard 6890N) coupled to a mass spectrometer (HP 5973) to obtain ion profiles for identifications. Oven temperatures were the same as described in De Troch et al. [12].

FAMES identification was based on the comparison of the mass spectral fragmentation spectra and retention times to authentic standards and mass spectral libraries (WILEY and in-house libraries) using the MSD ChemStation software (Agilent Technologies). Linear regression of peak areas of external standards (Supelco #47885, Sigma-Aldrich) in known concentrations ranging from 5 to 250  $\mu\text{g ml}^{-1}$  were used for FAME quantification. The slope of the linear regression of the external standards and amount of hexane is employed to quantify each individual FAME according to De Troch et al. [12]. Ratios between individual FAME and C16:0 peak areas were determined and ratios  $<0.02$  were deemed unreliable and, therefore, removed [16]. In addition, unassigned peaks were removed from the dataset. The nomenclature of the fatty acids follows the universal X:Y $\omega$ Z form, where X represents the number of carbon atoms, Y equals the number of double bonds and Z gives the position of the double bond counting from the terminal methyl group [26]. Because we were unable to reliably distinguish isoforms and anteisoforms, these peaks, of each respective FA, were summed and “(ante)iso” was added to the universal X:Y $\omega$ Z form throughout the rest of this paper. FAME concentrations were standardized to the total amount of nematodes added, resulting in pico grams per nematode. Because organisms have the ability for a genotype to match their phenotype to different environments which can influence the total amount of FAs under different experimental conditions, relative proportions of each quantified FAME to the total amount of pg fatty acids were determined. Similarly, relative FA proportions of the *E. coli* food source (added to experimental microcosms) were determined as described above.

#### Statistical analysis on *Halomonhystera hermes* and GD1<sub>p</sub>

To test the difference in relative fatty composition between *Halomonhystera hermes* and GD1<sub>p</sub>, a one factor permutational based multivariate analysis of variance [5], on the basis of Bray–Curtis similarity with 9999 permutations, was performed. The results were visualized by non-metric multidimensional scaling (MDS). Samples were clustered based on group average and a SIMPER analysis was used to identify which fatty acid accounted for the observed dissimilarity between species. PERMDISP [4] was executed to test the homogeneity of multivariate dispersions. Because of the limited level of replication, a Monte Carlo test was performed at all times and all p values in this study have been FDR-corrected in R version 3.0.2 [54].

Comparing relative proportions of FAs based upon the total fatty acid content between species might not truly reflect the composition of SFAs, MUFAs and PUFAs. Therefore, fatty acids were divided into three classes

based on the saturation level: saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFAs) and poly-unsaturated fatty acids (PUFAs). The total values for each class are the sum of values for the following major components: SFAs (C12:0, C13:0, C14:0, (ante)isoC15:0, C15:0, (ante)isoC16:0, C16:0, (ante)isoC17:0, C17:0, C18:0, C20:0 and C22:0), MUFAs (C14:1 $\omega$ 5, C15:1 $\omega$ 5, C16:1 $\omega$ 7, C17:1 $\omega$ 7, C18:1 $\omega$ 12, C18:1 $\omega$ 9, C18:1 $\omega$ 7, C20:1 $\omega$ 9, C22:1 $\omega$ 9 and C24:1 $\omega$ 9) and PUFAs [C16:2 $\omega$ 6, C18:2 $\omega$ 6, C18:3 $\omega$ 3, C18:3 $\omega$ 6, C20:2 $\omega$ 6, C20:3 $\omega$ 6, C20:4 $\omega$ 6, C20:5 $\omega$ 3 (EPA) and C22:6 $\omega$ 3 (DHA)]. Relative values of each FA were determined based upon the total sum of its respective FA class. The difference in SFA-, MUFA- and PUFA-composition, was statistically analysed as described above (Bray–Curtis similarity). The difference between both species of each FA proportion per class was determined using Euclidean distances with 9999 permutations and a PERMANOVA was performed.

In addition, PUFAs with a chain length  $\geq 20$  carbons and  $\geq 3$  double bonds, known as HUFA, were summed, SFA/UFA ratio (with UFA defined as the sum of MUFA and PUFA) and the unsaturation index were determined. The unsaturation index is the sum of MUFA and PUFA proportions multiplied by the respective amount of unsaturated bonds of each respective FA. To test the difference between SFA/UFA ratios, unsaturation indices and HUFA proportions, Euclidean distances was used in PERMANOVA. All analyses were performed within PRIMER v6 with PERMANOVA+ add-on software [5]. Figures were made with Graphpad prism 5.

#### Statistical analysis of the experiment (GD1)

Relative proportions of FAs were determined in the same way as described above and the same parameters were calculated. We have performed a fully crossed PERMANOVA design with three fixed factors (on all parameters separately: total FA-, SFA-, MUFA-, PUFA- and HUFA-composition, SFA/UFA ratios and unsaturation indices). The three factors were temperature (16 and 4 °C), salinity (25 and 34) and sulphide (0 and 1 mM). Because we lack proper replication, we cannot reliably estimate factor interaction effects and, therefore, pooled the two- and three-way interaction terms, thereby overestimating the true residual. This statistical test allowed us to reliably investigate the effect of each individual factor.

## Results

#### FA composition of GD1<sub>p</sub> and *H. hermes*

The total fatty acid composition was significantly different between both species ( $p = 0.0054$ ), and homogeneity of multivariate dispersions was found ( $p = 0.1044$ ). Relative values for all FAs, based upon the total FA



content can be found in Table 1. Replicate samples from *H. hermes*i clustered together with a similarity of >90 % and GD1<sub>p</sub> replicates clustered together with a similarity >80 % (Fig. 1). Total FA composition between both species was 68.65 % similar. SIMPER revealed that MUFA C16:1 $\omega$ 7 was present in significantly higher proportions ( $p = 0.0032$ ) in *H. hermes*i (23.56 %) compared to GD1 (9.07 %) and was most important in explaining the dissimilarity (23.11 %) between both species. This analysis further revealed that a total of 17 FAs contributed to explain >90 % of the dissimilarity (Table 2).

Classifying each FA into their respective group (SFAs, MUFAs and PUFAs) showed that MUFAs were the most abundant class for both species followed by PUFAs and SFAs (Fig. 2). SFAs C13:0 and ante(iso)C17:0, and MUFA C15:1 $\omega$ 5 were only observed in GD1<sub>p</sub> (Table 1; Fig. 3a). However, C13:0 was only identified in one GD1<sub>p</sub> sample in very low proportions (0.13 %). SFA C22:0, MUFAs C14:1 $\omega$ 5 and C18:1 $\omega$ 12, and PUFAs C16:2 $\omega$ 6, C18:3 $\omega$ 6, C20:3 $\omega$ 6 and C22:6 $\omega$ 3 (DHA) were exclusively observed in *H. hermes*i (Fig. 3b, c). Of these uniquely observed FAs, DHA had the highest relative proportion (4.23 %) of the total fatty acid content (Fig. 3c). The unsaturation index and SFA/UFA ratio of GD1<sub>p</sub> was respectively lower ( $p = 0.0021$ ) and higher ( $p = 0.0032$ ) in GD1<sub>p</sub> compared to *H. hermes*i (Table 3).

The relative amount of SFAs for *H. hermes*i (8.85 %) was significantly lower than the 28.43 % for GD1<sub>p</sub> ( $p = 0.0023$ ). C16:0 was the dominant SFA (Fig. 3a) in both GD1<sub>p</sub> (31.0 %) and *H. hermes*i (42.5 %). In addition, SFA composition was significantly different between both species ( $p = 0.0017$ ). Except for C13:0 ( $p = 0.40557$ ), C15:0 ( $p = 0.26365$ ) and ante(iso)C16:0 ( $p = 0.05720$ ) all SFAs were significantly different between both species ( $p$  values <0.05, Additional file 1: Table S1). C14:0 ( $p = 0.0102$ ) and ante(iso)C15:0 proportions ( $p = 0.0023$ ) were significantly higher in GD1<sub>p</sub>, while C16:0, C17:0, C18:0 and C20:0 were significantly higher in *H. hermes*i (Fig. 3a).

The low relative SFA proportion in *H. hermes*i was compensated by significantly higher MUFA—(11.83 %,  $p = 0.0030$ ) and PUFA (7.75 %,  $p = 0.0392$ ) proportions in comparison to GD1<sub>p</sub> (Fig. 2). The MUFA composition in both species was dominated by C16:1 $\omega$ 7 and C18:1 $\omega$ 9 (Fig. 3b) which were responsible for 67.1 and 79.9 % of the total MUFA composition of GD1<sub>p</sub> and *H. hermes*i, respectively. The MUFA composition was significantly different between both species ( $p = 0.0387$ ). MUFAs C18:1 $\omega$ 9, C18:1 $\omega$ 7, C20:1 $\omega$ 9, C24:1 $\omega$ 9 were not significantly different, while all other MUFAs were significantly different between both species ( $p$  values <0.05, Additional file 1: Table S1). The MUFA composition of *H. hermes*i consisted of circa two times higher proportions

of C16:1 $\omega$ 7 ( $p = 0.0102$ ) compared to GD1<sub>p</sub> (Fig. 3b), and was responsible for 40.2 % of the MUFA dissimilarity between both species.

PUFA compositions were also significantly different between both species ( $p = 0.0023$ ) and were dominated by C18:2 $\omega$ 6 (Fig. 3c), responsible for 40.14 % of the dissimilarity. The PUFA composition of *H. hermes*i showed a tendency towards HUFAs ( $\geq 20$  carbons,  $\geq 3$  double bonds) as was indicated by significantly higher HUFA values ( $p = 0.0032$ ) for *H. hermes*i (Table 3). This was a result of higher EPA (C20:5 $\omega$ 3—EPA;  $p = 0.0017$ ) proportions and the presence of DHA (C22:6 $\omega$ 3—DHA), a HUFA that was absent in GD1<sub>p</sub> (Fig. 3c; Table 1). EPA and DHA were responsible for 11.72 and 9.29 % of the PUFA dissimilarity between both species.

#### FA composition of GD1 under different experimental conditions

Similar to GD1<sub>p</sub>, SFAs had the lowest proportions in all experimental microcosms (Fig. 4; Table 1). SFA proportions were significantly affected by temperature ( $p = 0.0368$ ) resulting in 9.9–14.6 % lower SFA proportions at 4 °C compared to 16 °C (Fig. 4). Similar increases in UFA proportions were observed (Fig. 4) when temperature decreased ( $p = 0.0069$ ). Thus, the SFA/UFA ratio always significantly decreased ( $p = 0.0074$ ) with decreasing temperatures while the unsaturation index showed an inverse response ( $p = 0.0444$ , Table 3). Increasing the salinity and adding sulphides did not have a significant effect on SFA proportions.

SFAs mainly consisted of (ante)isoform FAs and odd chain length FAs such as C15:0 and C17:0 (Fig. 5a) and the most abundant MUFAs were C16:1 $\omega$ 7 and C18:1 $\omega$ 7 (Fig. 5b). The effect of varying abiotic factors did not reveal any discernible trend in SFA and MUFA composition. PUFA compositions were very similar and highly dominated by C18:2 $\omega$ 6 (71.5–80.7 %) which is similar to GD1<sub>p</sub> (67.4 %) and *H. hermes*i (59.3 %). The PUFA composition (Fig. 5c) of experimental nematodes lacked C18:3 $\omega$ 3, compared to GD1<sub>p</sub>, DHA was not observed in the experimental nematodes and only trace amounts of EPA (<1 %) were present.

#### FA composition of *E. coli*

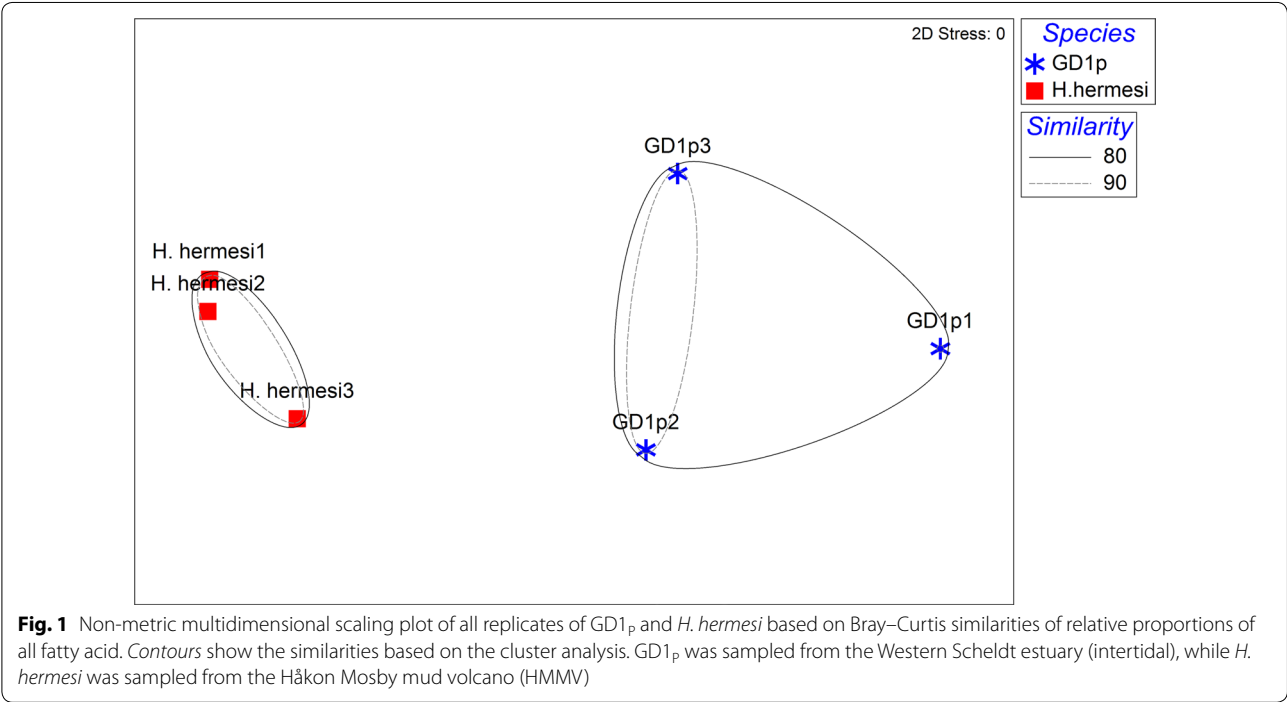
The most abundant fatty acids for the *E. coli* food source were SFAs (93.5 %) and no PUFAs could be identified (Table 1). SFA composition was similar to experimental nematodes (Fig. 5a) and was dominated by (ante) isoC15:0 (37.3 %) and C16:0 (29.4 %). Meanwhile, the composition of the MUFAs was very different from experimental GD1 nematodes (Fig. 5b) and consisted of C16:1 $\omega$ 7 (32.0 %), C18:1 $\omega$ 9 (51.1 %) and C18:1 $\omega$ 7 (16.8 %).



Table 1 continued

	Sampled for their original habitat		GD1 under different experimental culture conditions								Food source
	GD1 <sub>p</sub>	<i>H. hermes</i> <sub>i</sub>	25–16 °C—NoS	25–4 °C—NoS	25–16 °C—S	25–4 °C—S	34–16 °C—NoS	34–4 °C—NoS	34–16 °C—S	34–4 °C—S	<i>E. coli</i>
C18:2ω6	19.4 (±3.31)	15.95 (±0.45)	20.64	28.83	28.02	31.38	21.18	32.73	32.45	35.36	–
C18:3ω3	1.47 (±0.75)	2.51 (±0.55)	–	–	–	–	–	–	–	–	–
C18:3ω6	–	1.72 (±0.52)	–	–	–	–	–	–	–	–	–
C20:2ω6	4.33 (±0.37)	4.05 (±0.16)	3.31	6.55	5.89	6.58	2.17	7.79	7.53	6.75	–
C20:3ω6	–	0.21 (±0.02)	–	–	–	–	–	–	–	–	–
C20:4ω6	2.99 (±0.38)	3.53 (±0.22)	2.23	4.59	2.94	3.63	2.77	3.44	3.24	2.32	–
C20:5ω3(EPA)	1.29 (±0.24)	3.90 (±0.23)	–	0.37	–	0.20	0.13	0.23	0.41	–	–
C22:6ω3(DHA)	–	4.23 (±0.43)	–	–	–	–	–	–	–	–	–
Σ PUFA	29.49 (±3.97)	37.24 (±1.15)	26.19	40.35	36.84	41.80	26.24	44.20	43.63	44.43	0

Mean values (±SD) of GD1<sub>p</sub> and *H. hermes*<sub>i</sub> are represented. GD1 experimental labels consist of the salinity (25 or 34)—temperature (4 and 16 °C)—presence of sulphides is indicate by NoS (absence of sulphide) or S (presence of sulphide). Anteiso- and iso-form from a single fatty acid were summed and indicated by the addition of (ante)iso before each respective fatty acid



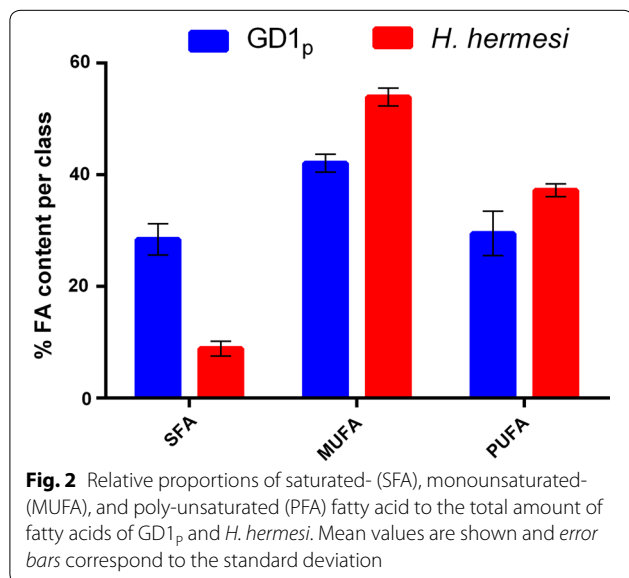
Discussion

Fatty acid compositions of *H. hermes*<sub>i</sub> and GD1<sub>p</sub> indicate a homeoviscous adaptation to the deep-sea environment

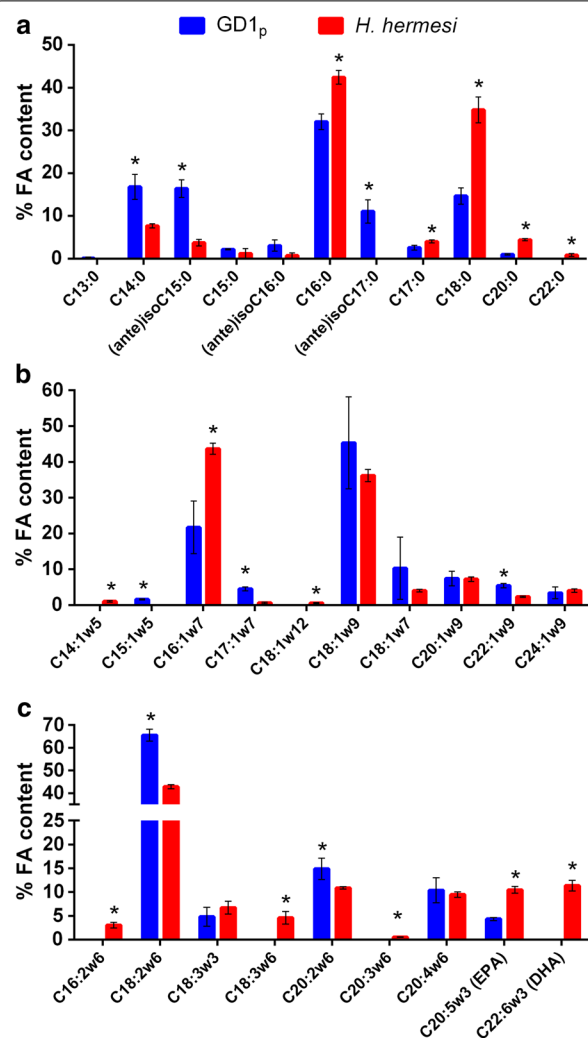
Survival and successful reproduction of organisms relies on the ability to respond and adapt to the often varying conditions imposed by their environment. Fatty acid

**Table 2** Contribution (%) of fatty acids to the total fatty acid content dissimilarity (up to 90 %) between GD1<sub>p</sub> and *H. hermes*

Fatty acid	Contribution (%)	Cumulative (%)
C16:1ω7	23.11	23.11
C16:0	8.55	31.65
C18:1ω9	7.20	38.85
(ante)isoC15:0	6.92	45.77
C22:6ω3 (DHA)	6.75	52.52
C14:0	6.60	59.12
C18:2ω6	5.87	65.00
(ante)isoC17:0	4.98	69.97
C20:5ω3 (EPA)	4.15	74.13
C18:1ω7	3.66	77.79
C18:3ω6	2.74	80.53
C17:1ω7	2.50	83.04
C16:2ω6	1.82	84.86
C18:3ω3	1.77	86.63
C22:1ω9	1.66	88.29
C18:0	1.65	89.95
C20:1 ω9	1.35	91.29



composition is known to rapidly (24–72 h) respond to changes in diet [66] and even daily/hourly fluctuations in FA composition have been observed previously [18, 23]. The barrier function of the cytoplasmic membrane is known to depend critically on the physical state of lipid bilayers [11], making it susceptible to environmental changes. The deep sea is a constant cold environment. When biological membranes are sufficiently cooled many biochemical reactions may be impaired which results in



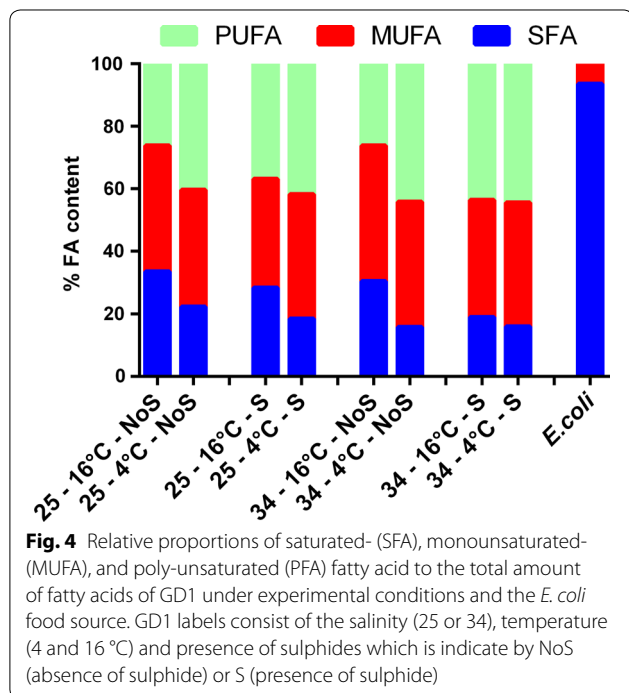
a decreased fluidity of lipids, a factor of primary importance to cell membrane function [30]. Increased MUFA proportions are most commonly reported as the agents involved in maintaining membrane order, with the addition of further double bonds having little effect on the phase transition properties and hence fluidity of the lipids [29]. Higher 16:1ω7 proportions in *H. hermes* could, therefore, be important in HVA to low temperatures. However, the high 16:1ω7 proportions could also have originated from the deep-sea HMMV bacteria *Beggiatoa* sp., the proposed food source for *H. hermes* which is rich in 16:1ω7 [72]. This MUFA has formerly been reported



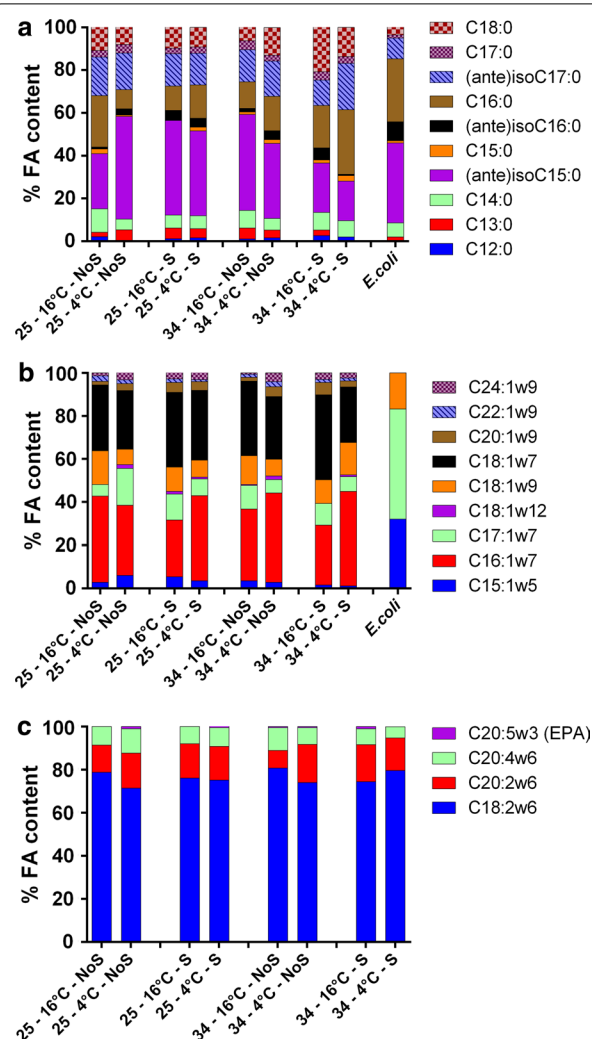
**Table 3 SFA/UFA ratio, unsaturation index and highly unsaturated fatty acid (HUFA) proportion of GD1 (GD1<sub>p</sub>) and *H. hermesii* sampled from their natural environment, GD1 under different conditions of salinity (25 and 34) temperature (16 and 4 °C), and presence of sulphide (NoS = absent and S = presence), and of the *E. coli* food source**

Treatment—species	SFA/UFA	Unsaturation index	HUFA
GD1 <sub>p</sub>	0.40 (±0.06)	1.12 (±0.07)	4.28 (±0.16)
<i>H. hermesii</i>	0.10 (±0.02)	1.68 (±0.03)	11.78 (±0.88)
GD1 (25–16 °C—NoS)	0.50	0.97	8.52
GD1 (25–4 °C—NoS)	0.29	1.28	12.30
GD1 (25–16 °C—S)	0.40	1.14	7.97
GD1 (25–4 °C—S)	0.23	1.31	9.17
GD1 (34–16 °C—NoS)	0.44	1.02	11.02
GD1 (34–4 °C—NoS)	0.19	1.36	8.31
GD1 (34–16 °C—S)	0.23	1.32	8.37
GD1 (34–4 °C—S)	0.19	1.33	5.22
<i>E. coli</i>	14.41	0.06	0

SFA proportion of saturated fatty acid, UFA proportion of unsaturated fatty acids, HUFA proportion of highly unsaturated fatty acids defined as unsaturated fatty acids with a chain length  $\geq 20$  carbon atoms and  $\geq 3$  double bonds



in *Beggiatoa* species from other cold seeps [20], suggesting that high 16:1w7 proportions might be important in HVA to cold-seep environments for *Beggiatoa* and in



extension also for organisms feeding on the bacteria such as *H. hermesii*. Low temperatures can also result in an increase in PUFA proportions in nematodes [63], which can explain the increase in PUFA proportion observed in *H. hermesii*.

High hydrostatic pressure can have similar effect on FA composition as low temperatures, i.e. reduction in SFA proportion and an increase of UFA proportion [6, 13, 14, 82]. Lipids are known to be more compressible than proteins and are, therefore, in comparison more sensitive to pressure effects [78]. Accordingly, many deep-sea organisms modulate their membrane fluidity and composition in response to pressure [6]. Comparison of deep-sea and shallow-water fish revealed increased proportions of membrane fluidizing unsaturated fatty acids [9]. An

increase in MUFAs and PUFAs and decrease in SFAs was also observed in vertically migrating marine planktonic copepods with increasing depth [52]. Our results revealed very similar effects in FA proportions, i.e. the proportion of saturated FAs in the deep-sea nematode *H. hermes*i is nearly 20 % lower compared to its intertidal relative GD1<sub>p</sub>, which is compensated by higher amounts of MUFAs and PUFAs.

Fatty acid proportions of *H. hermes*i revealed the presence of the highly unsaturated fatty acid (HUFA) DHA (DHA—C22:6 $\omega$ 3), which was absent in GD1<sub>p</sub>, and higher concentrations of the HUFA EPA (EPA—C20:5 $\omega$ 3). Marine animals are known to contain high levels of these long-chain  $\omega$ 3 PUFAs [2, 65] and recent data suggest that also marine nematodes are rich in HUFAs [42] as was observed for *H. hermes*i. HUFAs are essential for normal functioning of cell membranes and membrane-bound enzyme systems, crucial regulators for animal cell membrane physiology [8]. Moreover, these HUFAs were hypothesized to be important in HVA to high pressure and low temperatures [81]. DHA proportions also increased with depth in vertically migrating copepods suggesting its key role in maintaining membrane order in cold, high pressure conditions in the deep sea [52]. The absence of DHA in GD<sub>p</sub>, contrasting to its presence in *H. hermes*i, therefore, suggests that DHA might be essential in HVA to a deep sea environment. Moreover, interaction between temperature and pressure on DHA proportions, consistent with homeoviscous adaption, has been recently reported [52].

Because both species are bacterivorous, one could expect DHA and EPA to originate from their respective food source. Even though DHA is uncommon in most bacteria, it is present in deep-sea bacteria, as is EPA [14, 83]. Studies on deep-sea bacteria [13, 50, 81] and growth experiments of bacterial cultures under high pressure [35] revealed the importance of increased proportions of the HUFA EPA (EPA—C20:5 $\omega$ 3) under high pressure, which is in agreement with our results. However, a trophoecological study on fatty acid compositions of *Beggiatoa* sps, the proposed food source of *H. hermes*i, revealed that HUFAs were absent [72]. Unlike most metazoans, nematodes are, however, able to biosynthesize HUFAs from acetate [7, 56] and from shorter fatty acid precursors [32]. Interestingly, a transcriptome comparison between GD1<sub>p</sub> and *H. hermes*i [70] disclosed an overrepresentation of the KEGG pathway “fatty acid elongation” in *H. hermes*i. Given the absence of these EPA and DHA in the food source of *H. hermes*i [72], the nematode has most likely the ability to synthesize these HUFAs. EPA and DHA in addition can be assimilated into triglyceride forms and stored until they are required for incorporation in the membrane, beta-oxidation,

signaling activity, etc. [55, 79]. It, therefore, remains to be investigated what the primary function of these HUFAs is in *H. hermes*i.

It must be noted that other environmental differences might influence the FA composition of membranes. High salinities, for example, have an opposite effect on FA composition compared to high pressure and a low temperature [3, 38, 49]. A recent study revealed that increased salinities can also result in increased DHA and EPA proportions [21]. Moreover, synchronous effects of temperature, hydrostatic pressure, and salinity on FA composition have been previously observed [37]. Given the different (a)biotic features between both environments and the similar effect of temperature and pressure on the FA composition it is difficult to assess which abiotic factor is most responsible for the higher UFA proportion in *H. hermes*i. Therefore, we have experimentally tested the effect of different factors (temperature, salinity and sulphides) on the FA composition of GD1 to elucidate which factors significantly affected UFA proportions.

#### FA composition of GD1 is affected by temperature, but not by salinity and sulphide changes

Our results revealed that reduced growth temperatures result in greater proportions of unsaturated fatty acids, which is in accordance with previously observed temperature effects in nematodes [33]. However, no effect of salinity and sulphide was observed. This indicates that temperature is most likely the main factor responsible for the difference in UFA proportions between GD1<sub>p</sub> and *H. hermes*i, while salinity and sulphides are of lesser importance. Our results further revealed that DHA was absent in all our experimental nematodes and only trace amounts of EPA have been found. This suggests that the high HUFA proportions of *H. hermes*i, in comparison to GD1<sub>p</sub>, is most likely a response to other factors such as pressure and less the result of differences in temperature, sulphides and salinities between both environments.

Our experimental data further showed high proportions of C15:0 and C17:0, and especially high (ante) isoform proportions of several FAs. Metazoa almost exclusively synthesize even-chain fatty acids [24] while bacteria are able to synthesize odd chain length, ante- and iso-forms of FAs, and branched fatty acids [36]. The presence of C17:0 and C15:0 has been previously observed in bacterivorous nematodes, be it in low amounts [32], in agreement with our study. It is, however, well established that the fatty acid composition of an organism most often reflect their diet [45, 57, 72]. Meanwhile, transfer up the food chain of C15:0 and C17:0 have been seen in copepods feeding on bacterivorous ciliates [19] and an unusually high amount (up to 50 %) of odd chain length FAs

have been observed in the amphipod *Pontoporeia femorata* [51]. Both observations suggest that an organism is able to incorporate bacterial FAs. Odd chain length FAs and ante(iso) FAs are indeed present in the *E. coli* food source for GD1 and reflect the SFA composition of GD1 suggesting that the latter incorporates fatty acids from its food source to use in its membranes. In addition, absence of PUFAs in *E. coli* does clearly indicate and confirm that nematodes are able to biosynthesize long unsaturated fatty acids from shorter chain fatty acids as has previously been observed [59].

## Conclusion

In this study we have shown that the fatty acid composition of closely related deep-sea and intertidal nematodes (GD1<sub>p</sub>) clearly differs. The deep-sea nematode *H. hermes*i had higher unsaturated fatty acid proportions which could be attributed to the cold deep-sea environment. In addition, the saturated-, monounsaturated-, and poly-unsaturated fatty acid composition of both species is different. *H. hermes*i showed significantly higher 16:1ω7 proportions, which might be important in HVA to cold-seep environments. The deep-sea nematode had elevated HUFAs such as EPA and DHA, suggesting that these HUFAs are important in HVA to the cold deep-sea environment as was previously reported for other deep-sea organisms. Moreover, the lack of DHA and trace amounts of EPA in our experimental microcosms suggest that these HUFAs are a response to factors such as pressure and not a consequence of differences in temperature, salinity and sulphide concentrations. Subjecting GD1 to changing abiotic factors such as temperature, salinity and sulphides, revealed that only temperature significantly increased the unsaturated fatty acid proportions. This indicates that temperature is important in explaining differences in UFA proportions between GD1<sub>p</sub> and *H. hermes*i. Finally, we were able to show that nematodes have the ability to synthesize polyunsaturated fatty acids which are not present in their food source.

## Additional file

**Additional file 1: Table S1** Closely related intertidal and deep-sea *Halomonhystera* species have distinct fatty acid compositions.

## Authors' contributions

JVC and AV conceived the study; JVC performed the experiment, sampling and all the analyses and data handling. All authors contributed to data interpretation. JVC wrote the manuscript with significant input of AV. All authors read and approved the final manuscript.

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## Competing interests

The authors declare that they have no competing interests.

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